

OSTEOARTHRITIS and CARTILAGE

Chondrocyte tumor necrosis factor receptors and focal loss of cartilage in osteoarthritis

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Summary

Objective: Osteoarthritis (OA) is characterized by focal loss of cartilage. Here we show for the first time that tumor necrosis factor (TNF) α can act on cartilage but only at specific sites where chondrocyte TNF α -receptor (R) expression is high.

Design: Cartilage explants from specified sites in the knee joints of both OA patients and non-arthritic (NA) subjects were cultured with and without TNF α for 14 days and cumulative glycosaminoglycan (GAG) release into the supernatant measured. p55 and p75 TNF-R expression was measured by flow cytometry on chondrocytes isolated from the same sites.

Results: Cartilage explants from different sites in knee joints from both OA patients and NA subjects varied in their susceptibility to TNF α . Overall, the proportion of samples that responded to TNF α was higher in cartilage taken from OA joints than cartilage from NA subjects. Variations in p55 and p75 TNF-R expression were found between chondrocytes from different sites. p55, but not p75 TNF-R, expression on chondrocytes was closely related to the susceptibility of explants from the same site to TNF α -induced GAG loss.

Conclusion: It is considered that focal loss of cartilage will occur at sites where chondrocyte p55 TNF-R expression is high, if sufficient TNF α is present, and that these results identify a mechanism by which cytokine-mediated focal loss of cartilage may occur.

Key words: Osteoarthritis, TNF α , TNF-receptors, Chondrocytes, Human articular cartilage.

Introduction

DEGRADATION of cartilage matrix and the eventual loss of functional joint cartilage are features of both osteoarthritis (OA) and rheumatoid arthritis (RA), although the anatomical distribution of such loss differs between the two diseases. Since the discovery by Fell and Jubb of a factor from porcine cartilage which stimulated chondrocytes to degrade cartilage [1] and its subsequent characterization as the cytokine interleukin-1 (IL-1) [2, 3], attention has focused on the possible role of cytokines in these diseases. It is now generally accepted that locally produced cytokines, particularly IL-1 and tumor necrosis factor (TNF) α are major stimulants of cartilage degradation in RA. This view is based on the fact that IL-1 and TNF α are produced spontaneously by RA synovium [4–6], and that both cytokines can degrade animal cartilage *in vitro* [7, 8] and

in vivo [9, 10]. Despite the latter reports it remains to be established whether cartilage catabolic cytokines contribute to the process that leads to loss of cartilage in OA, although some observations lend credence to this theory. For example, cells from OA synovium produce higher amounts of IL-1 β [11] than normal synovium, TNF α is secreted by OA synovium [12], and both these cytokines have been detected in synovial fluid from OA patients [13–15]. In addition, the overall activity of cartilage-degrading enzymes, known to be produced by chondrocytes in response to stimulation by IL-1 or TNF α , is increased in OA [16]. However, a major difficulty for the cytokine theory is to explain how cartilage is lost only at particular anatomical sites, i.e., how can cytokines mediate focal loss of cartilage in OA?

We showed some years ago that OA cartilage explants appeared to be more susceptible to the degradative effects of IL-1 [17] and TNF α [18] than nonarthritic (NA) cartilage. We also showed that OA chondrocytes expressed more of the signal transducing p55 TNF-R than NA chondrocytes [19] whilst others provided evidence that type I IL-1R expression is increased on OA chondrocytes [20]. Increased susceptibility to cytokine-induced

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degradation thus appears to be associated with increased cytokine receptor expression. If so, these results suggest that for cartilage degradation to occur, not only must the cytokines be present, but the cartilage must be susceptible to their effects. To explain how TNF α contributes to the focal loss of cartilage in OA this hypothesis predicts that cartilage from different anatomical sites in joints varies both in its susceptibility to TNF α -induced GAG loss and in the levels of chondrocyte TNF-R they express. Moreover, a relationship should exist between chondrocyte p55 TNF-R expression and the susceptibility of explants from the same area to TNF α -induced GAG loss. The purpose of this work was to determine whether or not these predictions are satisfied. Accordingly, this paper describes experiments in which TNF-R expression on chondrocytes from human articular cartilage at six anatomical sites on femoral condyles was measured and related to the susceptibility of the remaining explant portion to TNF α -induced degradation.

Materials and Methods

CARTILAGE

Cartilage was obtained less than 6 h post-operatively from specific anatomical regions of the knee joints (Fig. 1) of OA patients undergoing surgery for total knee replacement ($N=8$; four female and four male, mean age 66 ± 10.3 years, range 43–75 years). Details of joint damage were diagrammatically recorded: the cartilage on seven OA knees was damaged on the medial femoral condyle, whilst cartilage on the lateral side was macroscopically normal. Cartilage on one OA knee was damaged on the lateral condyle, whilst the medial side was intact. Cartilage was obtained less than 48 h post-mortem from the same regions of NA knee joints ($N=3$, two female, one male, mean age 69 ± 7.6 years, range 62–77 years) in patients with no clinical evidence of arthritis. This cartilage was macroscopically normal. Full-depth cartilage slices were removed, where available, under sterile conditions using a scalpel, washed twice in phosphate-buffered saline (PBS) and semi-circular biopsies cut using a 3 mm diameter biopsy punch.

CHONDROCYTE ISOLATION AND STAINING

For each region, offcuts from biopsied cartilage slices were finely chopped into cubes ($2-3 \text{ mm}^2$) and the chondrocytes released by sequential enzymatic digestion, as previously described [19]. The

resulting digest was filtered ($40 \mu\text{m}$, Falcon) to remove any debris and the chondrocytes washed in HAMS F12 medium supplemented with 2 mM L-Glutamine, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 25 mM HEPES and 10% fetal calf serum (FCS) (Complete HAMS). The viability of both the OA and NA chondrocytes as assessed by trypan blue exclusion was $91.4 \pm 5.5\%$ (range 78.7–99). To allow the TNF-Rs to return to pre-treatment levels [21, 22], chondrocytes were rested for 16 h (37°C , 5% CO_2) in flasks coated with 2% agarose (Type VII, Sigma) [19]. Next the chondrocytes were washed twice in 2 M sodium chloride/20 mM HEPES buffer (pH 7.4) and once in 2 M sodium chloride/20 mM sodium acetate (pH 4) to remove any bound ligand [19]. The viability of the chondrocytes was assessed again and was $73 \pm 12\%$. U937 cells, a monocytic cell line known to express TNF-R [23], were used as a positive control; Raji cells, a B-cell line reported not to express TNF-R [24], was used to determine nonspecific binding [25]. The chondrocytes and control cells were stained for p55 and p75 TNF-R expression using noncross-reacting antibodies [25] and analyzed by flow cytometry, as previously described [19]. Briefly, chondrocytes and control cells ($5 \times 10^5/25 \mu\text{l}$) were incubated (45 min, 4°C) with mouse monoclonal antibodies (kind gift of Dr M. Brockhaus, Hoffman La Roche, Basel) to either the p55 receptor (htr9), the p75 receptor (utr1), or with IgG₁ isotype control (Becton Dickinson). All cells were washed and incubated (15 min, 4°C) with goat anti-mouse IgG conjugated to fluorescein-isothiocyanate (Dako). The cells were then washed and resuspended in 250 μl of PBS/0.25% formaldehyde prior to analysis (FACScan, Becton Dickinson). Comparison was made between cells stained with specific monoclonal antibodies and cells from the same population stained with the appropriate isotype control. Results were calculated by determining the shift in fluorescence of the test population compared with control and expressed as the proportion of positive cells in the population analyzed (5000 cells). The reproducibility of the calculation of the percentage expression, the co-efficient of variance (CV), was $8.9 \pm 0.8\%$ and the limit of detection 2%.

To estimate the number of receptors per cell, beads exhibiting various numbers of mouse IgG molecules (Dako, Qifi-kit) were incubated, at the same time as cells, with fluorescein-labelled goat anti-mouse IgG. A standard curve of mean fluorescence intensity against antigen density was constructed using the beads. The net fluorescence

intensity of the chondrocytes run at the same setting was measured. The relative receptor number on the chondrocytes (which is directly proportional to the antigen density) was calculated from the standard curve. The reproducibility of the estimation of receptor number per chondrocyte (CV) was $8.7 \pm 1.0\%$ and the limit of detection 100 TNF-R per chondrocyte.

CULTURE OF CARTILAGE WITH TNF α

Six biopsies were taken from each region. To minimize variance each biopsy was cut in half and the two pieces placed in separate wells of a 48-well plate (Costar). One half of each biopsy was cultured in 1 ml RPMI supplemented with 5% normal human serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 mM HEPES (complete RPMI), and 10 ng rhTNF α (Kind gift of Dr G.R. Adolf, Bender, Vienna). The corresponding (control) halves were cultured in 1 ml complete RPMI alone. The biopsies were cultured for 14 days (37°C, 5% CO₂) and the media changed every 2 days. The culture supernatants from each time point were collected and stored at -20°C. At the end of the culture period each half biopsy was removed, dried in an oven (37°C, 1 week) and weighed.

The effect of TNF α on cartilage matrix was determined by measuring GAG released from the biopsy into the supernatant using the colorimetric method of Farndale [26]. The GAG content of the supernatants were adjusted for dry weight of biopsy and expressed as micrograms per milligram of cartilage. For cumulative GAG release from each half biopsy, the concentration of GAG released into the supernatants obtained at different time points were summated. Comparison was made between GAG release from cartilage cultured in the presence of TNF α and GAG release from cartilage cultured in RPMI alone. The results are expressed as percentage increase in GAG release,

$$\% \text{ increase} = \sum \frac{(\text{Test} - \text{Control})}{\text{Control}} \times 100.$$

STATISTICAL ANALYSIS

For the six biopsies from each region the Student's paired t-test was used to compare the difference in the amount of GAG released from the half cartilage biopsy cultured in the presence of TNF α with the corresponding control half cultured in medium. The difference in the proportion of OA and NA cartilage explants responding to TNF α was examined by χ^2 test.

Correlations between TNF-R expression and per cent increase in GAG release were assessed using Spearman's rank correlation co-efficient. $P \leq 0.05$ was taken as significant.

Results

To determine if cartilage from different regions within the knee joint varies in its response to TNF α cartilage was taken from different regions of the knee (Fig. 1), the explants cultured in the presence and absence of TNF α for 14 days and the total amount of GAG released into the supernatant measured. Fig. 2 details the results of experiments using explants from five OA [(a)–(e)] and three NA [(f)–(h)] patients. First, variation within patients can be seen in TNF α -induced GAG release from cartilage isolated from different regions of the knee. For example, the amount of GAG released by TNF α treated explants from regions 1, 3 and 4 of cartilage from patient a [Fig. 2(a)] were significantly higher than their untreated controls (60, 100 and 50% respectively). By contrast, insignificant amounts of GAG were released by explants from regions 2 and 6. Similarly, the amount of GAG released by explants from regions 1 and 5 of cartilage from NA subject (f) [Fig. 2(f)] were significantly higher than their controls (40, and 50% respectively) whereas insignificant amounts of GAG were released by explants from regions 2, 3 and 4.

Second, it can be seen that there was some variation in susceptibility to TNF α between explants from the same region in knees from

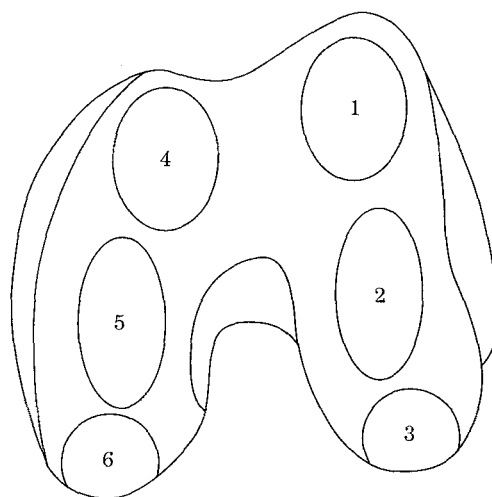


FIG. 1. Diagram of the different anatomical regions of the knee joint from which cartilage was obtained. Regions 1, 2 and 3 were located on the lateral femoral condyle, regions 4, 5 and 6 on the medial femoral condyle. Regions 1 and 4 were located at the anterior, regions 3 and 6 at the posterior aspects of the knee joint.

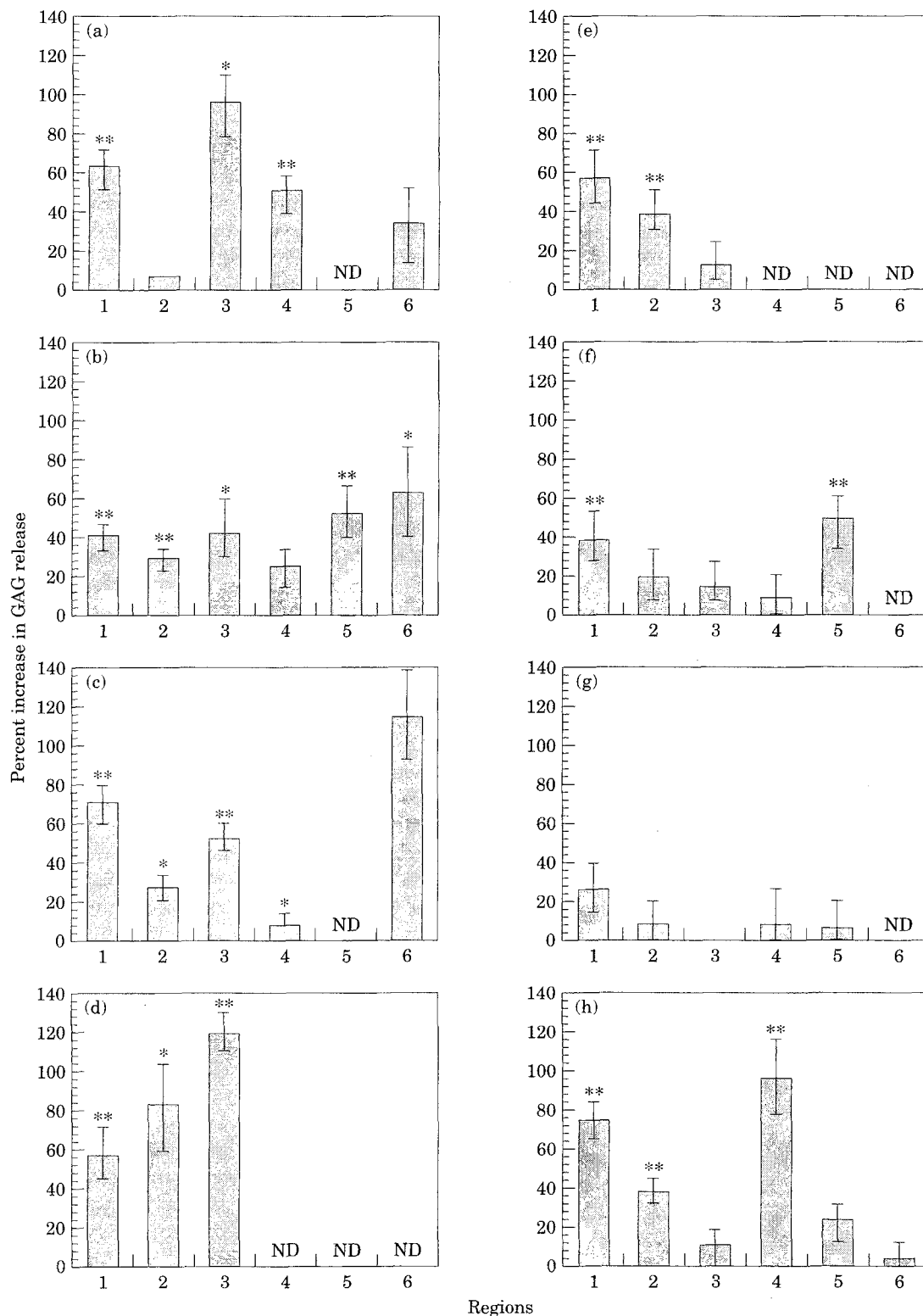


FIG. 2. Effect of TNF α on percentage GAG release during 14 days of culture from cartilage explants taken from specific anatomical regions of five OA knees, patients a, b, c, d and e, and three NA knees, patient f, g, and h. All values are the mean \pm SEM of six replicates. ND (not done) denotes regions where insufficient cartilage was available. Significant increase in TNF α induced GAG release as compared with control is indicated by ** $P < 0.01$ and * $P < 0.05$ (paired t-test).

different patients (compare regions 2, 3, 4 and 6 in patients a-h). Even in regions which were susceptible to $\text{TNF}\alpha$, the amount of GAG released as a proportion of the control varied (compare region 3 in patients a-e). Similar variations were observed with another three OA knees (results not shown).

The data in Fig. 2 shows the cumulative GAG released during 14 days of culture. The time required before a significant increase in GAG release was seen differed between regions and between patients. However, those explants which showed an early response to $\text{TNF}\alpha$ generally exhibited the greatest cumulative GAG loss by day 14. For example, (Fig. 3) in the joint removed from patient c, explants isolated from region 2 did not exhibit significant GAG release until day 10, whereas GAG release from region 6 reached significance after only 4 days.

Are explants from OA patients more susceptible to TNF -induced GAG loss than those from NA

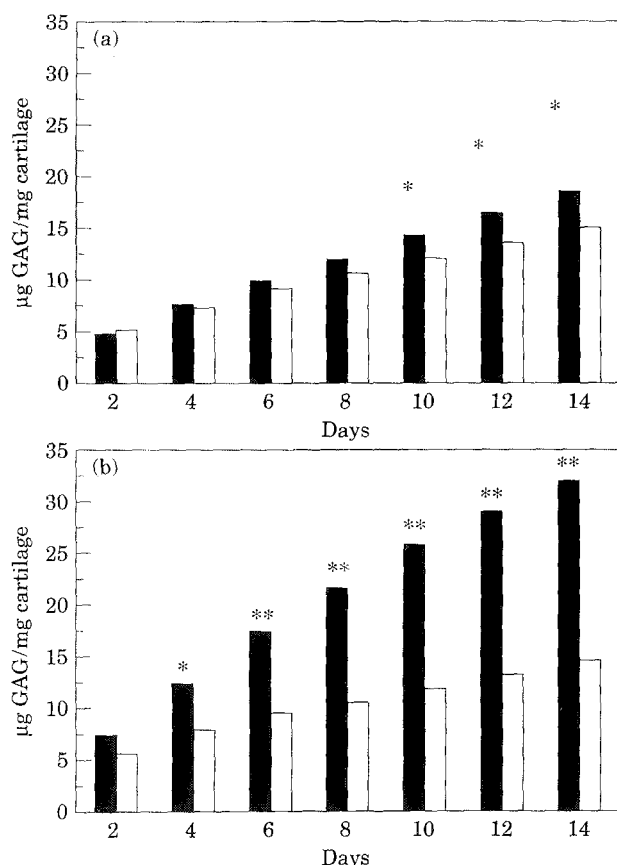


FIG. 3. $\text{TNF}\alpha$ induced cumulative GAG release from cartilage explants of patient c (a) region 2 and (b) region 6 over 14 days. Solid bars represent explants cultured with $\text{TNF}\alpha$ and open bars explants cultured in medium alone. Significant increase in $\text{TNF}\alpha$ induced GAG release as compared with control is indicated by ** $P < 0.01$ and * $P < 0.05$ (paired t-test).

subjects? Of the 33 areas sampled (six biopsies per sample) from specific regions of the OA knees, 25 (75.8%) exhibited significantly increased GAG loss in the presence of $\text{TNF}\alpha$ as compared with untreated controls. By contrast, only five of 15 areas sampled (33.3%) from different regions of the NA knees showed a significant TNF -induced increase in GAG loss as compared with untreated controls. The difference between the proportion of OA and NA explants responding to $\text{TNF}\alpha$ was significant ($P < 0.01$) as judged by the χ^2 test.

CHONDROCYTES TNF -R EXPRESSION

Experiments were performed to determine if there were also regional differences in the proportion of chondrocytes expressing TNF -R and the number of TNF -R expressed per chondrocyte. Accordingly, chondrocytes released from cartilage taken from the specified regions were stained for p55 and p75 TNF -R expression. It was found that both the proportion of chondrocytes expressing p55 TNF -R (Fig. 4) and the number of p55 TNF -R per chondrocyte (Fig. 5) differed between regions. For example, from Fig. 4(a) it is evident that 19 and 22% of chondrocytes isolated from regions 1 and 4, respectively, expressed the p55 TNF -R. By contrast, less than 4% of chondrocytes isolated from region 2 expressed the p55 TNF -R. Similarly, [Fig. 5(a)] chondrocytes isolated from regions 1 and 4 expressed between 4000 and 5000 p55 TNF -R/chondrocyte, whilst those from region 2 expressed less than 1000, and from region 3 and 6 expressed between 2000 and 3000 p55 TNF -R. Similar variations in TNF -R expression on chondrocytes isolated from different regions of NA knees were also found [Fig. 4 and Fig. 5(f-h)]. To determine whether the variability in chondrocyte p55 TNF -R expression could be attributed to cell viability immediately before analysis, a correlation was sought between the two parameters. No relationship was found for either proportion or receptor number ($RS = 0.19$, $N = 48$, $P = 0.35$ and $RS = 0.28$, $N = 48$, $P = 0.17$), respectively. A statistical analysis was performed to determine whether the number of p55 TNF -R/chondrocyte was related to the proportion of chondrocytes expressing p55 TNF -R. A positive correlation ($RS = 0.91$, $N = 48$, $P < 0.001$) was demonstrated.

As illustrated in Fig. 6 the proportion of chondrocytes expressing p75 TNF -R and the number of p75 TNF -R per chondrocyte again varied between different regions of the knee. However, chondrocyte p75 TNF -R expression was at or below the detection limit of the assay in many

regions and in some subjects (one OA and two NA) was at or below the detection limit in all regions (results not shown). A positive, although weak,

correlation was found between the proportion of chondrocytes expressing p55 and p75 TNF-R (RS = 0.51, $N = 48$, $P < 0.001$) and also between the

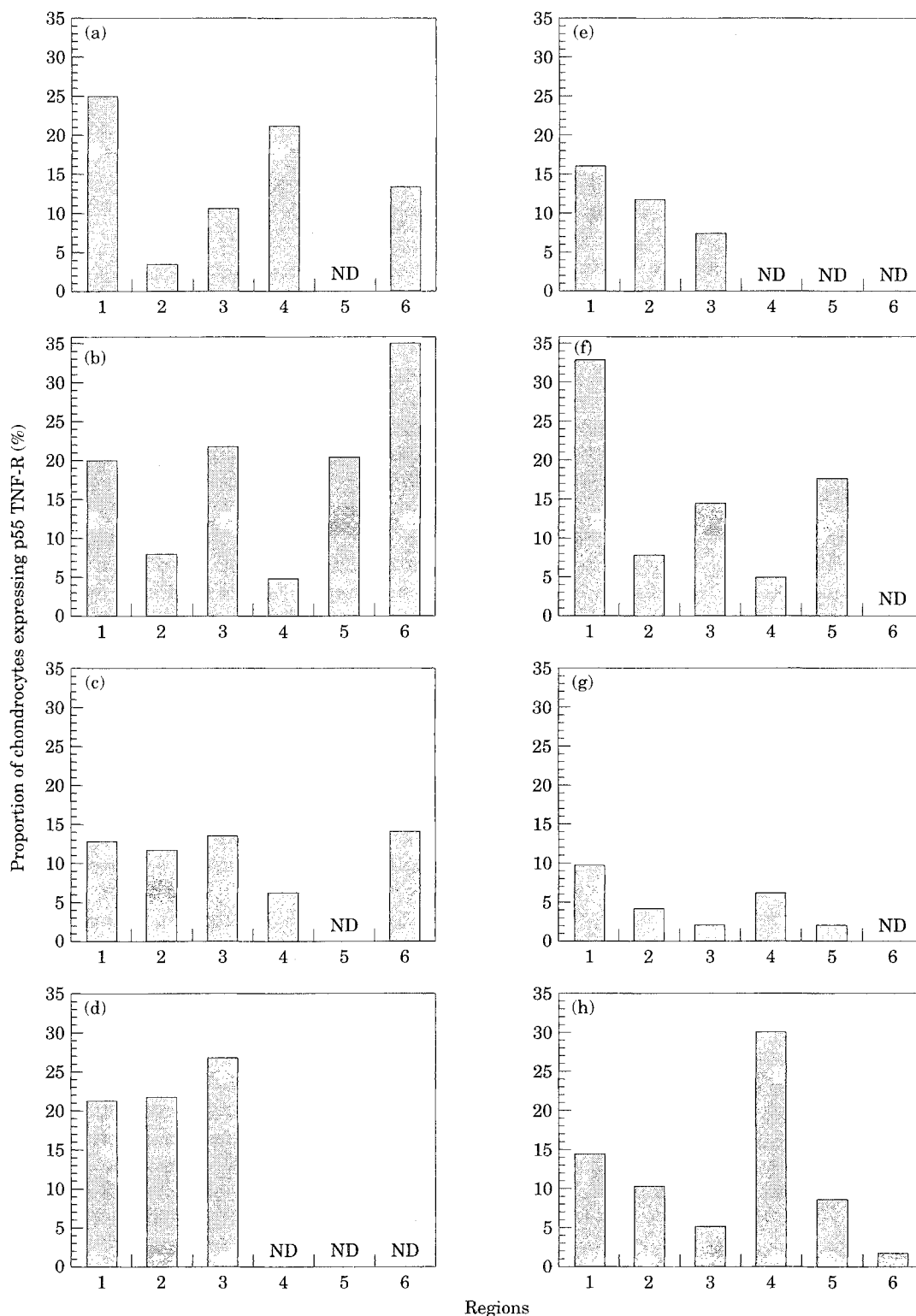


FIG. 4. Comparison of the proportion of chondrocytes from different regions expressing p55 TNF-R. Chondrocytes were isolated from the specific anatomical regions of five OA knees (patients a, b, c, d and e) and three NA knees (subjects f, g and h) and stained for p55 TNF/R expression. ND (not done) denotes regions where insufficient cartilage was available.

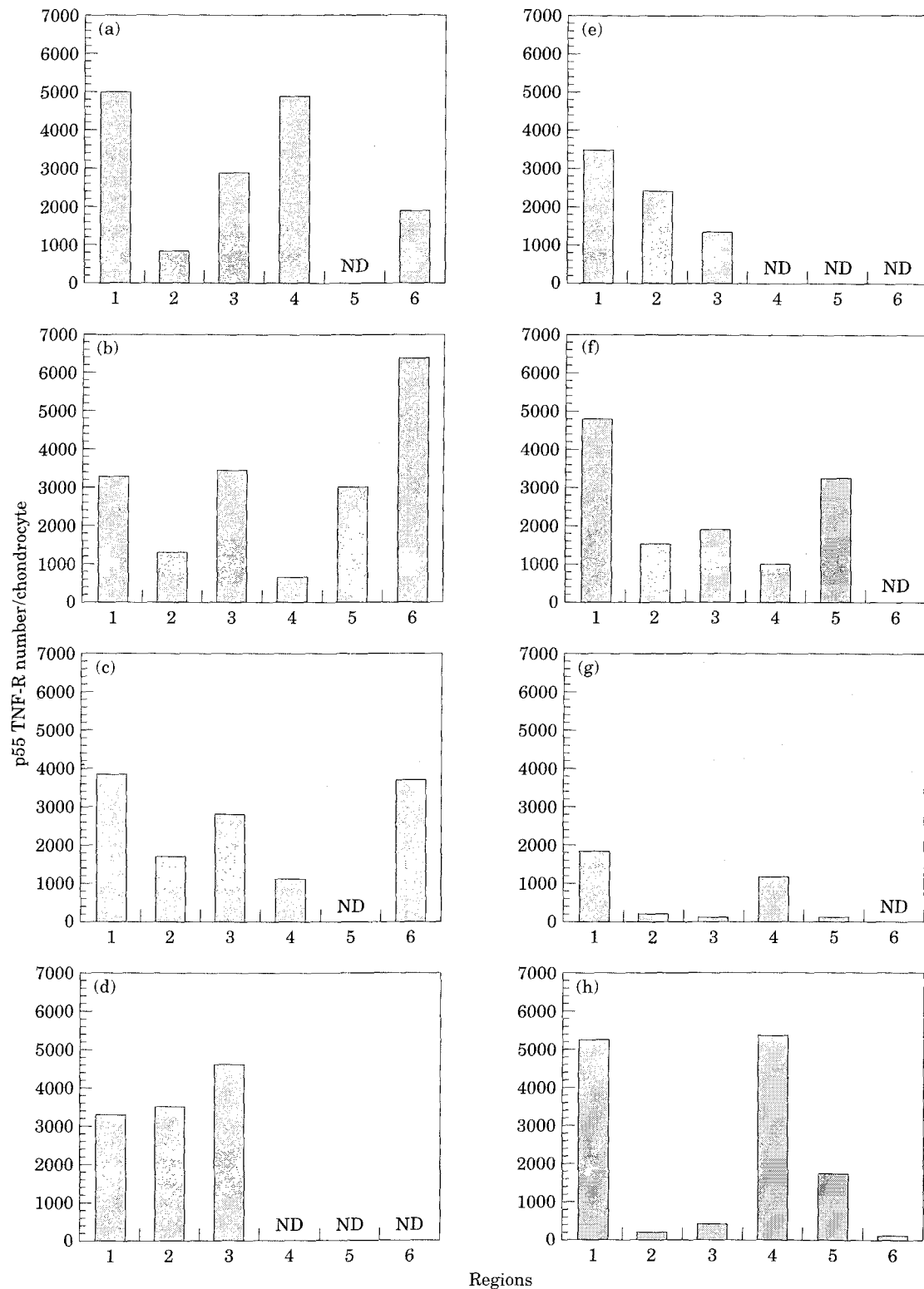


FIG. 5. Comparison of the relative number of p55 TNF-R on chondrocyte from different regions. Chondrocytes were isolated from the specific anatomical regions of five OA knees (patients a, b, c, d and e) and three NA knees (subjects f, g and h) and stained for p55 TNF/R expression. ND (not done) denotes regions where insufficient cartilage was available.

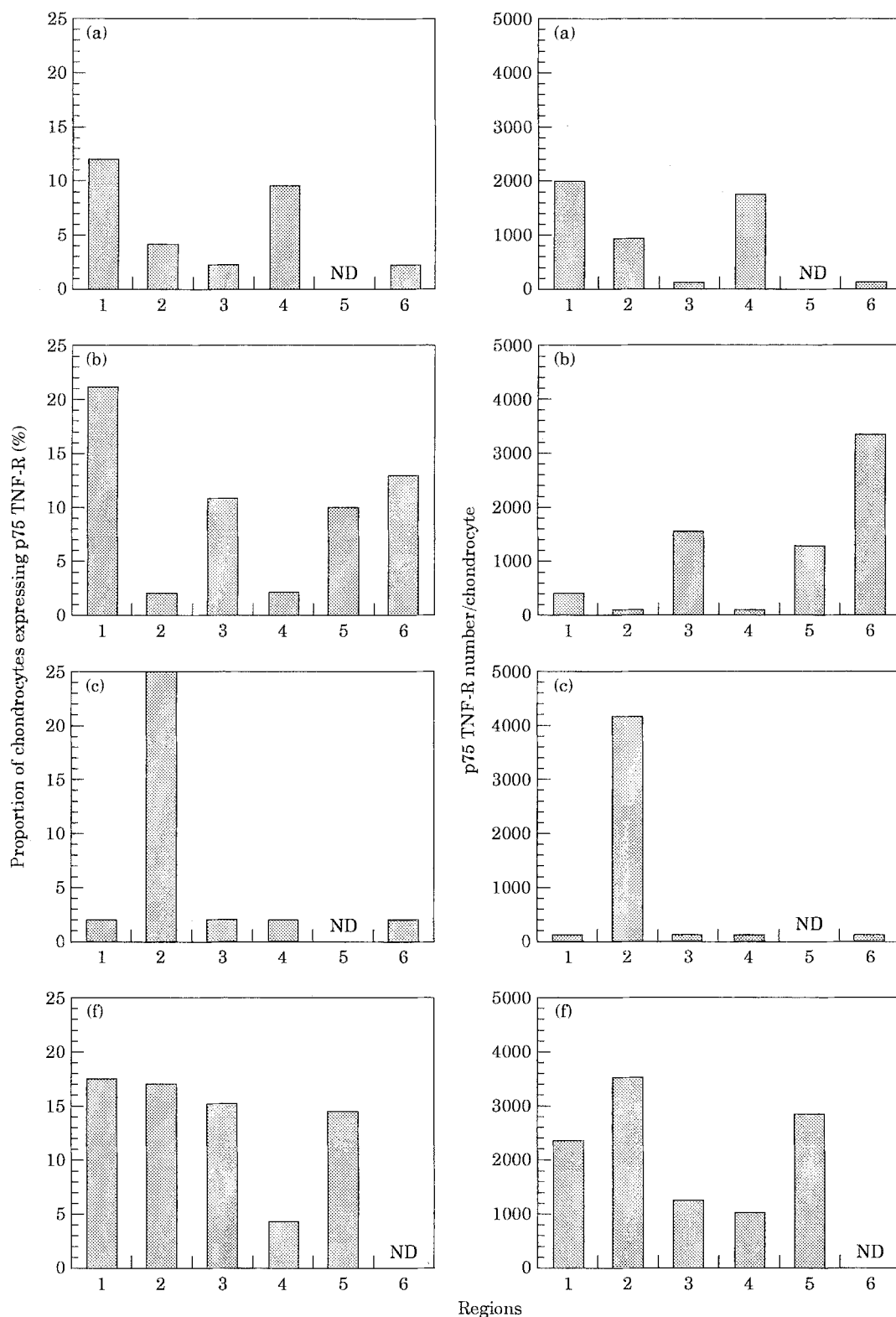


FIG. 6. Comparison of the proportion of chondrocytes expressing p75 TNF-R and the relative number of p75 TNF-R on chondrocyte from different regions. The results shown are for chondrocytes isolated from the specific anatomical regions of three OA knees (patients a, b and c) and one NA knee (subject f) stained for p75 TNF/R expression. ND (not done) denotes regions where insufficient cartilage was available.

number of p55 and p75 TNF-R/chondrocyte ($RS = 0.47$, $N = 48$, $P < 0.001$).

TNF-R EXPRESSION AND CARTILAGE DEGRADATION

If the variable response of cartilage explants to $TNF\alpha$ is due to the differential expression of functional p55 or p75 TNF-R then the amount of GAG released from the explants should be related to chondrocyte TNF-R expression. Fig. 7 shows that such relationships exist. Both the proportion of chondrocytes expressing p55 TNF-R and the number of p55 TNF-R/chondrocyte correlated with $TNF\alpha$ -induced GAG loss from cartilage explants. A significant relationship between these parameters was demonstrated whether OA and NA data were analyzed separately or combined. By

contrast no relationship was found between either the proportion of chondrocytes expressing p75 TNF-R ($RS = 0.12$, $N = 48$, $P = 0.4$) or the number of p75 TNF-R/chondrocyte ($RS = 0.15$, $N = 48$, $P = 0.33$) and $TNF\alpha$ -induced GAG loss from cartilage explants.

Discussion

These results show, for the first time, that the susceptibility of cartilage explants from different sites within the knee joints of OA patients and NA subjects varies in their response to $TNF\alpha$ as judged by $TNF\alpha$ -induced GAG loss. It is known that the superficial layers of normal human articular cartilage are more susceptible to stimulation by IL-1 than the deeper layers [27]. If the same is true for $TNF\alpha$, then it could be argued that for the cartilage from OA patients the observed regional variation results from the variable loss of the superficial zone at different sites. Since the zone most susceptible to catabolic cytokines would be lost, this argument suggests that OA cartilage explants should respond less well to $TNF\alpha$ than NA explants. This suggestion is not born out by the results. Overall a significantly greater proportion of OA explants responded to $TNF\alpha$ than NA explants, confirming earlier findings that pooled cartilage explants from OA patients exhibited enhanced response to $TNF\alpha$ as compared with explants from NA subjects [18]. An additional argument against the idea that the regional variations are due to variable loss of the superficial zone can be drawn from the fact that variations were found regardless of whether the explants were taken from the medial or lateral side of the cartilage, despite the fact that most of the patients were suffering from medial OA. Finally similar regional variations were observed with explants from NA subjects albeit on a limited number of individuals. Unfortunately no more cartilage specimens from NA subjects were available during the period of study.

It was found that not only did $TNF\alpha$ -induced GAG loss vary in different regions from the same knee, but there was some variation between explants from the same region in different knees. Although in total 48 explants in replicates of six were tested, the cartilage was obtained from only 11 individuals (eight OA, three NA). We do not therefore consider it possible to judge from these results whether explants from a specific region of knee joints was more susceptible to $TNF\alpha$ than any other.

The results show that a concentration of 10 ng/ml $TNF\alpha$ can consistently stimulate GAG

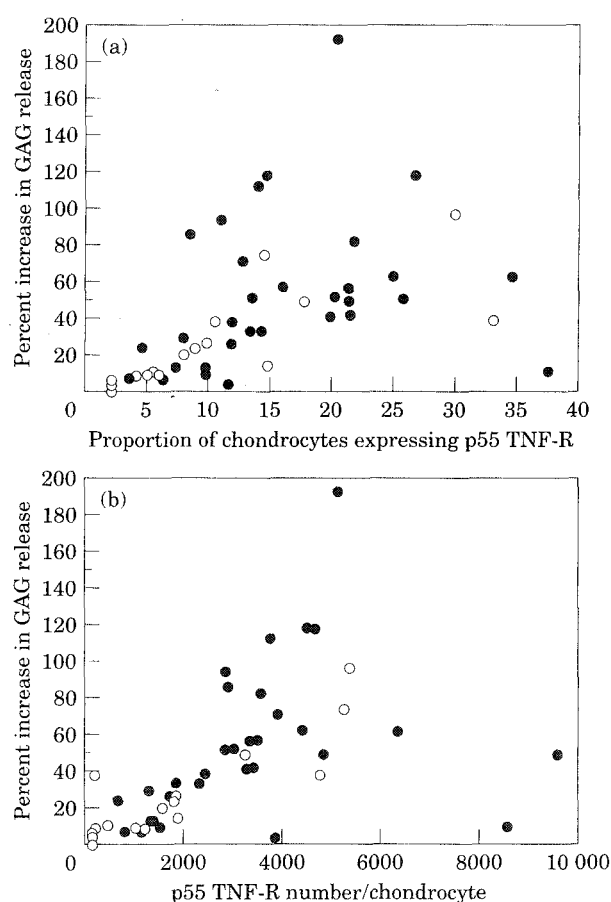


FIG. 7. Relationship between (a) cumulative $TNF\alpha$ induced GAG release after 14 days of culture and the proportion of chondrocytes expressing p55 TNF-R ($RS = 0.71$, $P < 0.001$, $N = 48$; OA $RS = 0.54$, $P = 0.002$, $N = 32$; NA $RS = 0.92$, $P < 0.001$, $N = 16$) and (b) cumulative $TNF\alpha$ induced GAG release after 14 days of culture and the relative number of p55 TNF-R/chondrocyte ($RS = 0.75$, $P < 0.001$, $N = 48$; OA $RS = 0.62$, $P < 0.001$, $N = 32$; NA $RS = 0.86$, $P < 0.001$, $N = 16$.) Closed circles represent explants from OA patients and open circles explants from NA subjects.

release from cartilage explants over a 14 day period. It may be asked if the concentrations of TNF α attained in OA joints are likely to reach this level. As a first step towards addressing this question we have taken 24 h culture supernatants from OA synovial membranes and measured TNF α concentrations. Work to be published elsewhere shows that of 35 OA membranes tested, several attained concentrations between 1 and 5 ng/ml over this time period. It remains to determine if similar concentrations of TNF α stimulate GAG release from human articular cartilage.

Variations were found both in the proportion of chondrocytes expressing TNF-R and the number of TNF-R/chondrocyte between chondrocytes from different regions. It should be noted that no relationship could be demonstrated between the viability of the chondrocytes before analysis and TNF-R expression, and that therefore variations in viability between chondrocyte preparations cannot explain the regional variation in receptor expression. Do the levels of chondrocyte TNF-Rs determine whether cartilage explants can respond to TNF? p55 TNF-R was suggested to be the major signal transducing molecule for TNF α [28]. If this is true for chondrocytes, it would be predicted that those in cartilage explants from sites that respond to TNF α should express more p55 TNF-R than those in explants from sites which failed to respond. The results do indeed satisfy these predictions. p55 but not p75 TNF-R expression on chondrocytes was closely related to the susceptibility of explants from the same site to TNF α induced GAG loss. This relationship held regardless of whether the data from OA and NA cartilage was analysed separately or combined. Taken together these results are consistent with the notion that if sufficient TNF α is present, only cartilage from those locations at which chondrocytes express high numbers of TNF-R will degrade. Thus these results suggest a mechanism by which focal loss of cartilage may occur in OA.

The results raise the question of how chondrocytes from different sites within joints vary in the levels of p55 TNF-R expression. One possibility is that variation in mechanical loading across the joint results in differential expression of cytokine receptors. This possibility gains credence from evidence of variations in susceptibility to IL-1 between chondrocytes from differently loaded joints: chondrocytes from normal human ankles were more resistant to IL-1 than those from knees of the same donors (H.J. Häuselmann, personal communication). It is well documented that alterations in mechanical loading change chondrocyte metabolism. For example, immobiliz-

ation of the knee results in a marked reduction in proteoglycan synthesis compared with the contralateral knee [29]. Different sites within a joint do vary in load-bearing [30] but to our knowledge there is no information regarding how load-bearing affects the expression of chondrocyte catabolic cytokine receptors. However, sheer stress can induce IL-6 mRNA expression in NA chondrocytes [31] and we have found that IL-6 can increase the expression of chondrocyte TNF-R [32]. Thus pathways by which mechanical loading can alter catabolic cytokine receptor expression may exist.

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